

THE EFFECT OF WATER CONTENT UPON THE RATE OF HEAT DENATURATION OF CRYSTALLIZABLE EGG ALBUMIN

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(Accepted for publication, May 29, 1933)

It has long been stated that proteins are much less readily denatured by heat when dried than when moist. This fact has given rise to the hypothesis (Chick and Martin, 1910) that denaturation is essentially a reaction between protein and water. It has also been used to explain the relative ineffectiveness for sterilization of dry as compared to moist heat (Rahn, 1932).

However, little experimental work has been carried out upon this interesting phenomenon, due no doubt to the difficulty of satisfactorily sampling a dry material at successive time intervals. Lewith (1890), at the suggestion of Hofmeister, powdered salt- and globulin-free egg albumin, dried it in a vacuum to various degrees of dryness, and, after sealing small samples in thin walled glass tubes, tested the effect of heating. He found that the "coagulation temperature" increased markedly with decreasing water content, being 56°C. in solution, 80–90°C. for protein containing 18 per cent water, and 160–170°C. for completely dry protein. Chick and Martin (1910) contributed the further information that when crystallizable egg albumin is heated in an air bath at 120°C., it remains completely soluble after 5 hours. At 130°C. it is slowly changed, so that after 4 hours 22 per cent is rendered insoluble in water. Hemoglobin behaves similarly.

We have repeated and extended the work of Lewith, in particular by relating the denaturation temperature to the relative humidity of water vapor with which the protein is in equilibrium rather than to its absolute water content. There are three advantages in this:

1. It is in general experimentally more convenient to bring a dry

protein or other material into equilibrium with a definite partial pressure of water vapor than to obtain a uniform sample of definite water content.

2. Whether or not the process of denaturation is assumed to be essentially a reaction between protein and water, it is obviously desirable to have a measure of water concentration or activity. The relative humidity (p/p_s) is the most suitable quantity for this purpose.

3. The results are in a form which may be directly compared with analogous experiments on the death rate of living organisms in atmospheres of various relative humidities. It is, of course, in general impossible to determine the actual quantity of water taken up by microorganisms.

In addition, however, we have determined the actual water content of the heated and unheated egg albumin corresponding to each value of the relative humidity, so that it is also possible to relate the denaturation temperature directly to that quantity. The necessary experimental data are given in the last section of this paper.

Technique

The technique employed was similar to that used by Lewith. Dry, crystallizable egg albumin was prepared by drying salt-free solutions over P_2O_5 in an electric refrigerator. The resulting material is white, readily soluble in distilled water except for a trace (< 2 per cent), and is capable of being recrystallized from $(NH_4)_2SO_4$ solution. Samples of this dry protein were placed in desiccators at room temperature over saturated salt solutions giving a wide range of relative humidities.

When the protein had come into equilibrium (as determined by constancy of weight) with water vapor at each relative humidity, three or four samples from each desiccator were placed in small tubes, sealed, and blown into a small bulb at one end. These tubes were made of 0.3 cm. thin walled glass tubing and were originally about 3.75 cm. long and of 0.25 cc. internal volume. The sample placed in each tube weighed from 5 to 10 mg. The open tubes were replaced in the desiccators from which the respective samples had been withdrawn and were left there for several days in order to insure continued equilibrium between protein and water vapor from the saturated salt solutions. Finally, the tubes were quickly sealed at such a distance as not to affect the egg albumin. The glass immediately in contact with the protein never became so warm that it could not comfortably be held. Samples were repeatedly tested and found to possess the same solubility as the original material.

The tubes of native egg albumin in equilibrium with atmospheres of various

relative humidities (p/p_s) so obtained were heated in an air thermostat at several temperatures to determine the range for each relative humidity in which the protein neither became completely insoluble nor remained entirely soluble in a definite time interval. Solubility was determined by observing the behavior of particles of the protein in a drop of distilled water on a microscope slide. Because of the extremely high temperature coefficient of denaturation, the range can be easily determined to within less than 10°C . The centers of the ranges determined for the various relative humidities are taken to correspond approximately to temperatures of equal denaturation velocity.

EXPERIMENTAL RESULTS

Tables I and II give the experimental data corresponding to heating times of 10 minutes and 60 minutes, respectively.

The data are summarized in Table III and presented graphically in Fig. 1.

It will be observed from Fig. 1 that the temperature of denaturation is very closely a linear function of the relative humidity. The points referring to $p/p_s = 0$ per cent and a heating time of 60 minutes, and to $p/p_s = 20$ per cent for both heating times fall furthest from the straight line. The latter deviation is undoubtedly due to the fact that the potassium acetate solution had not remained saturated, and therefore the humidity must have been too high. This fact also explains the apparent high value for the sorption of water at this relative humidity (see below). The former deviation (at $p/p_s = 0$ per cent) cannot be explained in this manner. According to the statement of Wichmann, thoroughly dry egg albumin remains soluble after heating at 150°C . for several hours. In our experiments, however, we could never obtain soluble protein at so high a temperature even after 1 hour. Several months after the main series of experiments was carried out, we repeated the tests at $p/p_s = 0$ per cent with egg albumin samples which were dried with special care over P_2O_5 . The temperature of denaturation was essentially the same as that previously found.

DISCUSSION

The experimental relation between relative humidity and the temperature of denaturation may be reduced to a more easily comprehensible form by assuming that the Arrhenius equation

$$\ln k = \frac{Q}{RT} + \text{constant}$$

TABLE I

Dependence of Temperature of Denaturation upon Relative Humidity. Time of Heating = 10 Minutes

Initially in equilibrium with	p/p_s 20°C.	Temperature of thermostat	Solu- bility*	Range of partial denaturation	Mid- point of range
	per cent	°C.		°C.	°C.
P ₂ O ₅	0	136.0-136.5	+++		
"	"	153 -154	+++		
"	"	158 -159	++-		
"	"	166 -167	---		
"	"	168 -169	---	158-166	162
LiCl·H ₂ O (saturated solution)....	15	136.0-136.5	+++		
" " "	"	145 -146	+++		
" " "	"	153 -154	---		
" " "	"	166 -167	---	146-153	150
KAc (saturated solution).....	20	128 -129	+++		
" " "	"	130.5-131.5	+++		
" " "	"	135 -136.5	---	130.5-135	133
CaCl ₂ ·6H ₂ O (saturated solution)...	32	113	+++		
" " "	"	114.5-115.5	+++		
" " "	"	121.5-122.5	+++		
" " "	"	127 -128	+±-		
" " "	"	131 -132	---	125-131	128
KCNS (saturated solution).....	47	105 -106	+++		
" " "	"	108.5-109.5	+--		
" " "	"	111.5-113	---	106-110	108
NaBr·2H ₂ O (saturated solution)...	58	90 - 91	+++		
" " "	"	95	+++		
" " "	"	97 - 98	+++		
" " "	"	100	+++		
" " "	"	104.5-105.5	+--	100-106	103
NaClO ₃ (saturated solution).....	75	81 - 82	+++		
" " "	"	84	+++		
" " "	"	91.5-92.5	---		
" " "	"	95	---		
" " "	"	97 - 98	---	85- 91	88

TABLE I—*Concluded*

Initially in equilibrium with	p/p_s 20°C.	Temperature of thermostat	Solu- bility*	Range of partial denaturation	Mid- point of range
	<i>per cent</i>	°C.		°C.	°C.
KBr (saturated solution).....	84	73 - 74	+++		
" " "	"	78 - 79	++-		
" " "	"	82	---		
" " "	"	90 - 91	---	75- 81	78
NH ₄ H ₂ PO ₄ (saturated solution)....	93	65 - 65.5	++-		
" " "	"	69.5- 70.5	+-		
" " "	"	73 - 73.5	---	65- 71	68
H ₂ O.....	Saturated	65 - 65.5	+±-		
"	"	69.5- 70.5	±--		
"	"	73 - 73.5	---	64- 71	67.5

* The pluses and minuses indicate the approximate solubility of the heated egg albumin in distilled water.

applies.¹ This assumption is justified since the Arrhenius equation is known quite generally to describe the dependence of the velocity of chemical reactions upon temperature. In particular, the denaturation of egg albumin in dilute solution follows this equation (Lewis, 1926).

An experimental relation which greatly facilitates the application of the Arrhenius equation is that the temperature coefficient (and the critical increment, also) of the denaturation reaction is, within the limits of error, independent of the relative humidity. This is merely another way of expressing the fact illustrated in Fig. 1 that the denaturation temperature: relative humidity curves, corresponding to heating times of 10 minutes and 60 minutes, are very closely parallel. It also follows, of course, that the reciprocal of the absolute temperature of denaturation is a linear function of p/p_s and the curves for the two heating times are parallel.

By means of the Arrhenius equation it is possible from a knowledge of the relative velocities of the denaturation process at the same relative humidity but at two different temperatures (Fig. 1) to calculate

¹ The author wishes to express his appreciation to Professor E. A. Guggenheim for pointing out this method of interpreting the experimental data.

TABLE II
Dependence of Temperature of Denaturation upon Relative Humidity. Time of
Heating = 60 Minutes

Initially in equilibrium with	p/p_s 20°C.	Temperature of thermostat	Solu- bility*	Range of partial denatura- tion	Mid- point of range
	per cent	°C.		°C.	°C.
P ₂ O ₅	0	133 -134	+++		
"	"	139 -140	+±-		
"	"	144 -145	---		
"	"	149 -150	---	137-143	140
LiCl (saturated solution)	15	128.5-130	+++		
" " "	"	133 -134	+±-		
" " "	"	140 -141	---	131-137	134
KAc (saturated solution)	20	120 -121	+++		
" " "	"	124 -125.5	+±-		
" " "	"	125 -126.5	---		
" " "	"	128 -129	---	122-126	124
CaCl ₂ (saturated solution)	32	111 -113	+++		
" " "	"	118 -119	+++		
" " "	"	120 -121	+±-		
" " "	"	124 -125	---		
" " "	"	125 -126.5	---	119-123	121
KCNS (saturated solution)	47	90.5- 91.5	+++		
" " "	"	95 - 96	+++		
" " "	"	99 -101	+±-		
" " "	"	111 -113	---	96-102	99
NaBr·2H ₂ O (saturated solution)	58	86 - 87	+++		
" " "	"	90.5- 91.5	+±-		
" " "	"	95 - 96	---		
" " "	"	99 -101	---	90- 96	93
NaClO ₃ (saturated solution)	75	70.0- 70.5	+++		
" " "	"	74 - 75	+±-		
" " "	"	80 - 81	±---		
" " "	"	86 - 87	---	73- 81	77
KBr (saturated solution)	84	59.0- 59.5	+++		
" " "	"	65 - 65.5	+±-		
" " "	"	70.0- 70.5	---		
" " "	"	74 - 75	---	62- 68	65
NH ₄ H ₂ PO ₄ (saturated solution)	93	51.5	+++		
" " "	"	56	+±-		
" " "	"	59 - 59.5	±---		
" " "	"	65 - 65.5	---	54- 60	57

* The pluses and minuses indicate the approximate solubility of the heated egg albumin in distilled water.

the relative velocity at any other temperature. This can be done for every experimentally determined temperature of denaturation corresponding to every relative humidity. Actually, absolute values of denaturation velocity have not been calculated but only the logarithms of the relative velocities referred to the velocity at 100°C. and $p/p_s = 58$ per cent as unity.

TABLE III
Summary of Data for Dependence of Temperature of Denaturation upon Relative Humidity

p/p_s	Temperature of half denaturation in 10 min.	Temperature of half denaturation in 60 min.
<i>per cent</i>	°C.	°C.
0	162	140
15	150	134
20	133	124
32	128	121
47	108	99
58	103	93
75	88	77
84	78	65
93	68	57

Fig. 2 illustrates the remarkable fact that the logarithms of the relative velocities at any definite temperature are a strictly linear function of the relative humidity, *i.e.*

$$\ln k = a \cdot p/p_s + b$$

or

$$k = c \cdot e^{ap/p_s}$$

where k is the denaturation reaction velocity constant, e is the base of the natural system of logarithms, p/p_s is the relative humidity, c is a constant dependent upon the temperature but independent of the relative humidity, and a and b are constants independent of both of these variables.

This is the first time to our knowledge that an exponential relation has been observed between the velocity of a chemical reaction and the concentration of one of the reacting substances. Although it is

evident that the concentration of water is of extreme importance in determining the velocity of denaturation, it is difficult to imagine what is the physical meaning of the exponential law. A possible

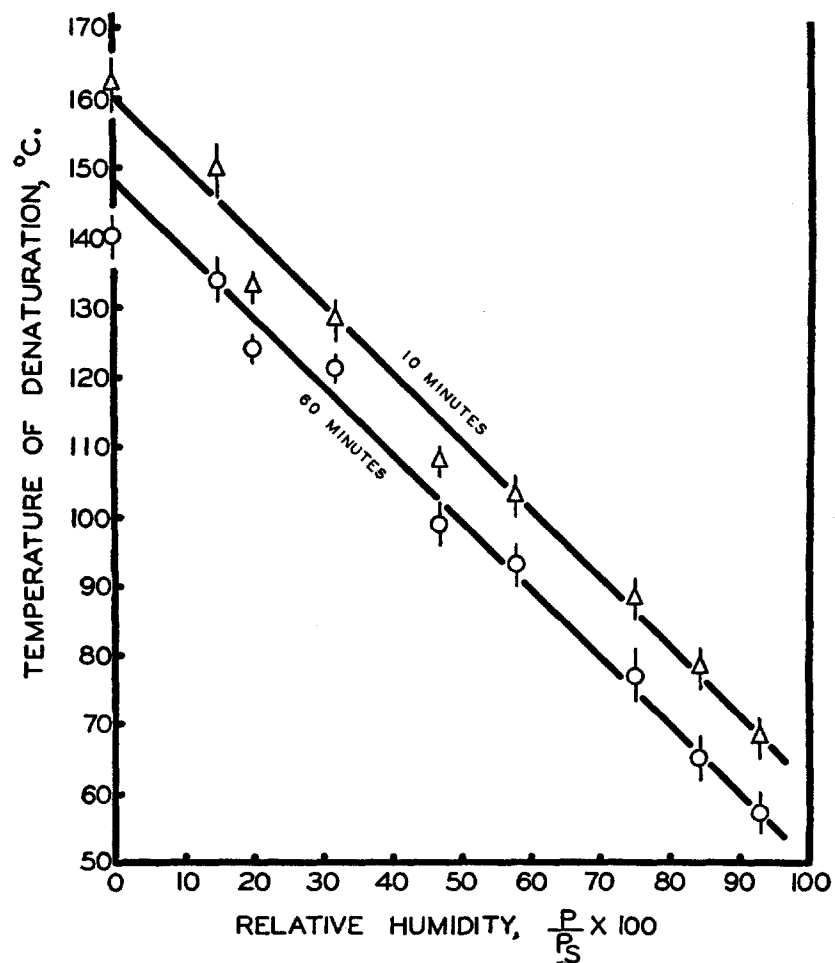


FIG. 1. The dependence of the temperature of denaturation of egg albumin upon the relative humidity of water vapor. Δ —experimental heating time 10 minutes; \circ —experimental heating time 60 minutes.

line of explanation is that the variation of the relative humidity not only alters the concentration and activity of the water, but also affects

the freedom of the water molecules to move between and upon the relatively immobile protein molecules and aggregates. The relative immobility of the water at low vapor pressure may be even more im-

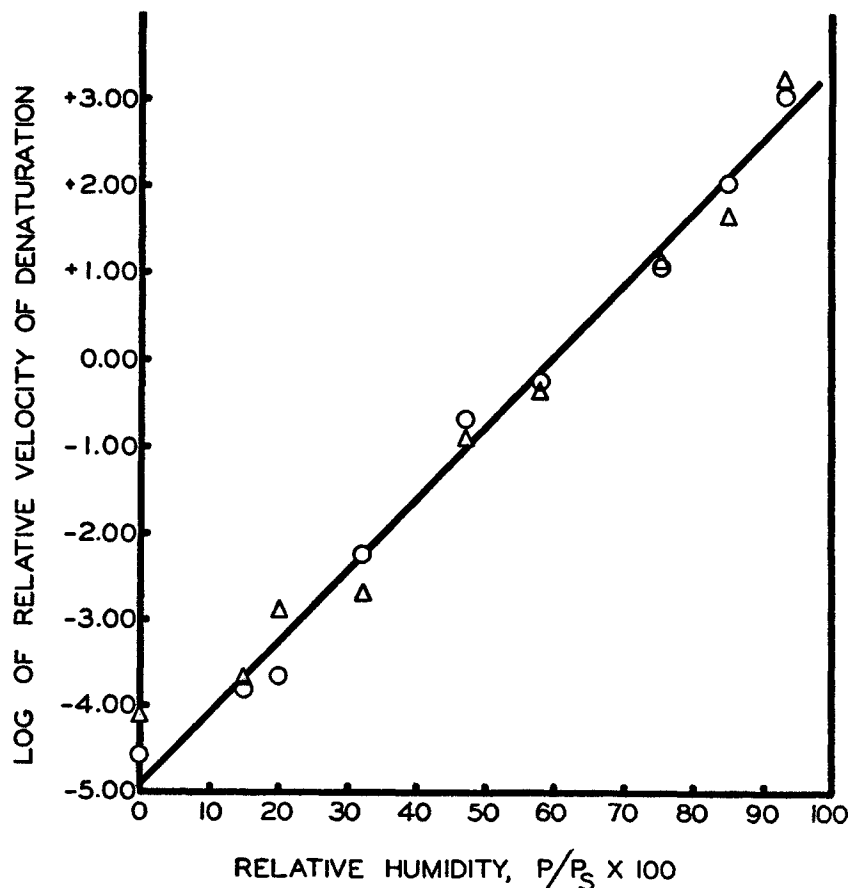


FIG. 2. Showing that the logarithm of the velocity constant of heat denaturation is a linear function of the relative humidity. ○—experimental heating time 10 minutes; △—experimental heating time 60 minutes.

portant in lowering the denaturation velocity than is the reduction in water concentration.

We have assumed that water actually reacts with the protein in the heat denaturation process. It must be admitted, however, that

our observations give no conclusive proof that this is true. It is conceivable that the indirect influences which we have just discussed can account for the entire importance of the water. Nevertheless it seems highly probable that the rôle of the water is also a direct one. These kinetic experiments must be considered to support this hypothesis.

We wish to point out that the observed relation between the temperature of denaturation and relative humidity affords a very direct method of studying the mechanism of thermal death in partially dried microorganisms such as bacterial spores and protozoan cysts. The effect of humidity is so great that its influence upon thermal death rate may be easily studied even in the presence of disturbing factors. And if it could be shown that the same relation also applies to this phenomenon, then that would constitute important evidence in support of the hypothesis that thermal death of dry microorganisms is due to protein alteration. Such experiments are in progress.

Water Vapor Pressure Isotherms of Native and Heat-Denatured Egg Albumin

As mentioned above, we have determined the water content of egg albumin as a function of the relative humidity over the entire range. These data are useful not only as a means of converting relative humidities into water contents and *vice versa*, but they also constitute a contribution to the question, so frequently discussed, of the gain or loss of water accompanying heat denaturation.

The sorption isotherms were determined by the desiccator method. Approximately 1 gm. of dry but soluble and crystallizable egg albumin was placed in each of thirty small weighing bottles. These were divided into two groups of fifteen each, one group being heated in a pressure steam sterilizer at 15 pounds per sq. inch for 10 minutes to give the denatured egg albumin, while the other group remained at room temperature. Half of the bottles of each group were brought to constant weight over P_2O_5 ($p/p_s = 0$ per cent), the other half of each group being similarly brought to equilibrium over a solution saturated with $NH_4H_2PO_4$ ($p/p_s = 93$ per cent). After this initial equilibrium had been attained, sorption experiments were carried out with one set of bottles by placing them in vacuum desiccators

over solutions of higher relative humidities, while the other set was placed at lower relative humidities for desorption experiments. In all experiments the anhydrous weight of the protein was determined as the final constant weight obtained over solid P_2O_5 which will undoubtedly agree closely with Sørensen and Høyrup's (1917) anhydrous protein dried to constant weight over solid potassium hydroxide.

1 or 2 weeks were required to attain constant weight. The change of weight was followed by weighings made on an analytical balance every 1 to 4 days. As soon as the desiccators were opened for weighings, the ground glass stoppers were placed on the weighing bottles contained therein.

We shall especially mention that the solubility and crystallizability of the dried native egg albumin were tested at intervals throughout the duration of the experiments. Every sample was found to be completely or almost completely (> 98 per cent) soluble in distilled water and almost all were recrystallizable from ammonium sulfate solution.

Table IV gives the experimental data which are represented graphically in Fig. 3.

The experimental data may be summarized by the statement that heat-denatured egg albumin takes up approximately 80 per cent as much water at each particular relative humidity as does native crystallizable egg albumin. This conclusion is in agreement with the less complete experiments of Sørensen and Sørensen (1925), Katz (1917), and Adair and Robinson (1931) as regards both the sign and the magnitude of this effect.

A comparison of the results of these sorption experiments with those from the study of the dependence of denaturation velocity upon relative humidity leads to the apparently anomalous conclusion that whereas water is necessary for denaturation and denaturation itself probably involves a reaction of the protein with water, yet the denatured protein is less heavily hydrated than the material from which it is derived. Even if the kinetic evidence demands the conclusion that the denaturation reaction actually involves a combination of water with the protein, it nevertheless can suggest nothing at all about the properties of the resulting protein. The quantity of water which might react would be too small to be analytically demonstrable

TABLE IV

Sorption of Water by Native and Denatured Egg Albumin. Temperature Range, 20–26.75°C. Mean Temperature, 23°C. x/m in Gm. Per Gm. of Anhydrous Protein

Protein in equilibrium with saturated solution of	$\frac{p}{p_0}$ 20°C.	Sorption		Desorption		Mean x/m		Ratio b/a
		Native	Dena- tured	Native	Dena- tured	Native	Dena- tured	
						(a)	(b)	
	<i>per cent</i>							<i>per cent</i>
LiCl·H ₂ O	15	0.0376		0.0453	0.0400	0.0417	0.0365	87
				0.0455	0.0377			
				0.0386	0.0294			
					0.0388			
KAc	20	0.0612		0.0682		0.0647		
CaCl ₂ ·6H ₂ O	32	0.0748		0.0839	0.0643	0.0808	0.0599	74
				0.0778	0.0550			
				0.0866	0.0604			
KCNS	47	0.0974		0.1067		0.1021		
NaBr·2H ₂ O	58	0.1183	0.0957	0.1235	0.1031	0.1247	0.1013	81
		0.1260	0.1009	0.1310	0.1054			
NaClO ₃	75	0.1637	0.1419	0.1744		0.1699	0.1322	78
		0.1695	0.1223					
		0.1722	0.1270					
			0.1378					
KBr	84	0.2058	0.1532	0.2102	0.1607	0.2053	0.1664	81
		0.1998			0.1788			
					0.1730			
NH ₄ H ₂ PO ₄	93	0.3143	0.2396			0.3059	0.2327	76
		0.3093	0.2259					
		0.3078						
		0.3006						
		0.3046						
		0.3059						
		0.3061						
		0.3090						
H ₂ O	Saturated	0.7495	0.3523			0.7366	0.3596	
		0.7237	0.3670					
Mean								79.5 per cent
Mean deviation								3.5 per cent

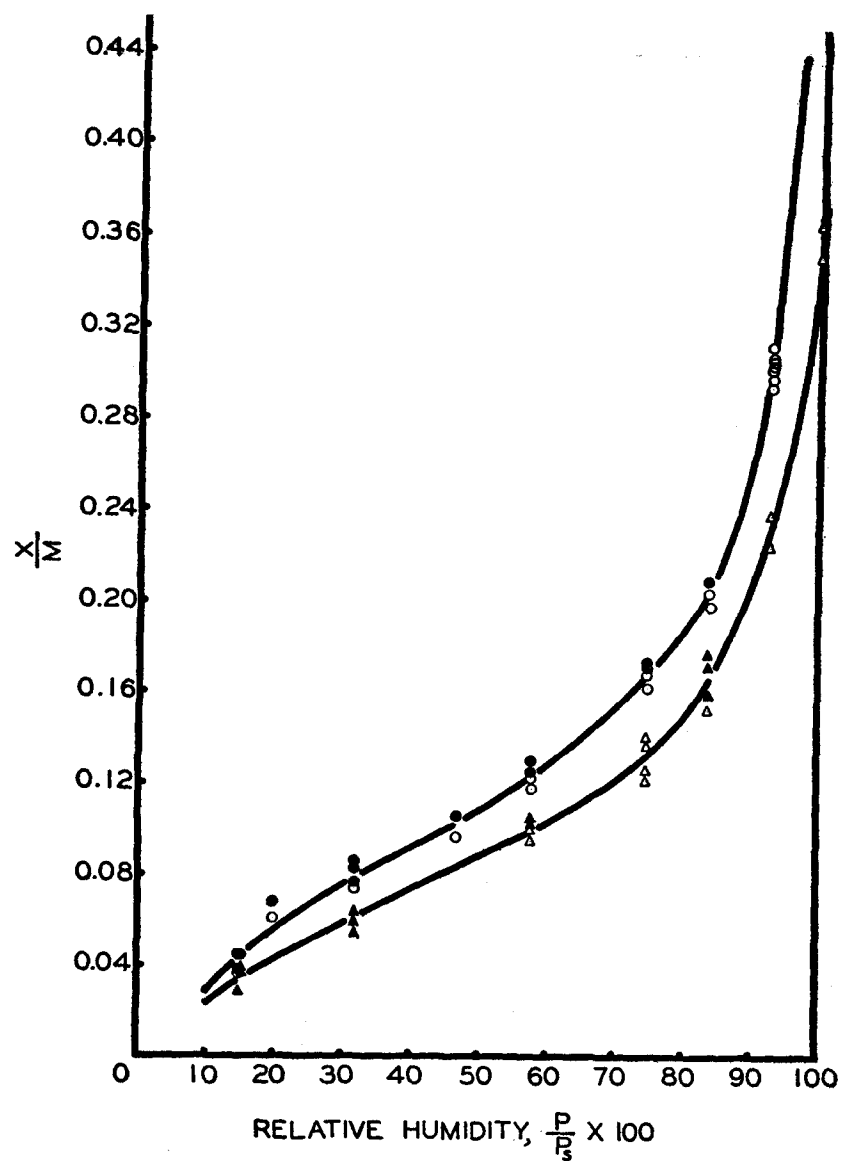


FIG. 3. Sorption of water by native and heat-denatured egg albumin. \circ —sorption, native; \bullet —desorption, native; \triangle —sorption, heat-denatured; \blacktriangle —desorption, heat-denatured.

(Hirsh-Pogany, 1922), and would be, moreover, entirely masked by the lesser affinity of the new protein derivative for water.

SUMMARY

1. The denaturation rate of partially dried crystallizable egg albumin is greatly decreased by decreasing its water content.

2. The temperature of denaturation, defined as the temperature at which half of the protein becomes insoluble in distilled water after a definite time of heating, is a linear function of the relative humidity with which the protein is in equilibrium.

3. By applying the Arrhenius equation it is shown that the rate of heat denaturation at a given temperature is an exponential function of the relative humidity.

4. The application of the observed relations to the analysis of the mechanism of thermal death of microorganisms is suggested.

5. The water content of native and heat-denatured egg albumin is determined as a function of the relative humidity of water vapor. It is shown that the heat-denatured modification takes up approximately 80 per cent as much water at all relative humidities as does native egg albumin.

In conclusion the author wishes to express his appreciation to Professor J. W. McBain for advice and criticism during the progress of this work.

REFERENCES

- Adair, G. S., and Robinson, M. E., 1931, *J. Physiol.*, **72**, 2P.
Chick, H., and Martin, C. J., 1910, *J. Physiol.*, **40**, 404.
Hirsh-Pogany, M., 1922, *Biochem. Z.*, **128**, 396.
Katz, J. R., 1917, *Kolloidchem. Beihefte*, **9**, 1.
Lewis, P. S., 1926, *Biochem. J.*, London, **20**, 965.
Lewith, S., 1890, *Arch. exp. Path. u. Pharmacol.*, **26**, 341.
Rahn, O., 1932, *Physiology of bacteria*, Philadelphia, P. Blakiston's Son and Co., Inc., 326.
Sørensen, S. P. L., and Høyrup, M., 1917, *Compt.-rend. trav. Lab. Carlsberg* **12**, 68.
Sørensen, S. P. L., and Sørensen, M., 1925, *Compt.-rend. trav. Lab. Carlsberg*, **15**, No. 9, 1.